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TITLE: The Endoplasmic Reticulum Stress Protein Calreticulin in Diabetic Chronic Kidney Disease

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
We hypothesize that ER stress induced by glucose in diabetes promotes diabetic CKD through CRT stimulation of TGF-beta-dependent calcium/NFAT signaling in renal proximal tubule cells. In Aim 1 we will determine the role of CRT in mediating the fibrogenic effects of TGF-beta and glucose in renal cells. In Aim 2, we will determine the role of CRT in mouse models of diabetic nephropathy. In year 2, we developed stably transduced HK-2 cells using lentivirus to enable us to perform long term studies in culture. Importantly, we performed animal studies this year, which showed that ultrasound mediated delivery of cre-recombinase plasmid to CRT floxed mice reduces CRT expression by ~30-40% with primary downregulation in the tubules which persisted over 5 months. Downregulation of CRT expression in diabetic CRT floxed mice showed a significant reduction in the urinary albumin/creatinine ratio, indicating attenuation of renal dysfunction, and improved survival. We also saw reduced fibrosis by electron microscopy and PAS staining and by western blot for fibronectin.

15. SUBJECT TERMS
Diabetic nephropathy, calreticulin, TGF-beta, endoplasmic reticulum stress, fibrosis

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1. INTRODUCTION

Rationale: ER stress promotes the development of diabetic nephropathy. The diabetic milieu increases ER stress through hyperglycemia, advanced glycation end products, oxidative stress, and decreased ER Ca^{2+} stores. While ER stress is initially adaptive, chronic stimulation leads to cell death and fibrosis. There is emerging evidence linking ER stress with fibrosis, but mechanisms are unclear. In diabetic CKD, transforming growth factor-beta (TGF-beta) stimulation of extracellular matrix (ECM) is central to development of diabetic CKD. *We recently identified the ER stress protein, calreticulin (CRT), as a critical regulator of TGF-beta stimulation of ECM, thereby establishing a mechanistic link between ER stress and fibrosis.* CRT is an ER chaperone and a regulator of ER calcium homeostasis and of nuclear factor of activated T cells (NFAT) signaling. High glucose and TGF-beta increase CRT in cultured proximal tubule cells. Increased CRT is observed in mouse models of tubulointerstitial fibrosis and in diabetic nephropathy. We showed that *CRT is required for TGF-beta transcriptional stimulation of ECM* and that CRT deficient cells fail to respond to TGF-beta ECM stimulation. CRT knockdown in human proximal tubule (HK-2) cells attenuates TGF-beta and high glucose stimulation of fibronectin and collagen I. CRT overexpression increases responses to TGF-beta. CRT mediates TGF-beta signaling through regulation of TGF-beta-stimulated calcium release and stimulation of NFAT activity. High glucose increases NFAT activity in HK-2 cells and NFAT blockade reduces diabetic renal fibrosis. *These data provide evidence that CRT is critical for TGF-beta and glucose stimulated ECM production, suggesting a new mechanism by which CRT drives ER stress-dependent diabetic CKD.* We hypothesize that ER *stress induced by glucose in diabetes promotes diabetic CKD through CRT stimulation of TGF-beta-dependent calcium/NFAT signaling in renal proximal tubule cells.* This will be tested in the following aims:

Specific Aim 1: To determine the role of CRT in mediating the fibrogenic effects of TGF-beta and glucose in renal cells Tubular injury and fibrosis are major determinants of progression to CKD. We will address whether CRT and/or NFAT regulate TGF-beta and glucose stimulation of the fibrotic phenotype in HK-2 cells by assessing ECM production, mesenchymal phenotype, migration, and resistance to apoptosis. We will use HK-2 cells subjected to CRT siRNA knockdown and proximal tubule cells isolated from CRT-floxed mice transduced with lentiviral Cre-recombinase. We will use NFAT inhibitors to address which responses are NFAT-dependent. Diabetes is an oxidative environment and hydrogen peroxide (H_2O_2), a mediator of oxidant signaling, can regulate TGF-beta dependent calcium signaling and ECM stimulation. Thus, we will address the potential role of CRT in regulating calcium release downstream of glucose/TGF-beta and H_2O_2.

Specific Aim 2: To determine the role of CRT in mouse models of diabetic nephropathy We will use several approaches to downregulate CRT to determine the role of CRT in diabetic CKD: we expect that CRT downregulation will attenuate renal fibrosis and improve renal function. **Aim 2a:** We will knockdown proximal tubule CRT in streptozotocin-treated CRT floxed B6D2F1mice using a proximal tubule promoter-specific (PEPCK) cre-recombinase plasmid delivered using kidney targeted microbubble/ultrasound-mediated plasmid delivery. We will also examine non-targeted CRT knockdown in these mice. **Aim 2.b**: We will similarly drive CRT knockdown using CRT RNAi plasmid delivery in the Akita/C57Bl6 uninephrectomy model of type 1 diabetes in which fibrosis is primarily tubulointerstitial. **Aim 2.c**: We will directly assess the role of NFAT in diabetic CKD through use of the NFAT inhibitor, 11R-VIVIT, in the Akita model. Both gene delivery and disease analyses will be performed with the assistance of the NIH-funded UAB O'Brien Center.

2. KEYWORDS: diabetes, chronic kidney disease, diabetic nephropathy, calreticulin, TGF-beta, ER stress, ultrasound, tubulointerstitial fibrosis

3. ACCOMPLISHMENTS

a. WHAT WERE THE MAJOR GOALS OF THE PROJECT?

*Tasks for year 2 (months 12-24) are highlighted in **bold***

Research-Specific Tasks:

Specific Aim 1: To determine the role of CRT in mediating the fibrogenic effects of TGF-β and glucose in renal cells	**Months**	**Progress towards completion**
Major Task 1: (Aim 1.1.a, 1.1b) Establish cell models of CRT knockdown in HK-2 and mouse proximal tubule cells	1-6	
Subtask 1: Confirm Not human subjects research for use of HK-2 cells through UAB IACUC and DoD HRPO Cell lines used: HK-2 [ATCC CRL-2190]	1-3	100%
Subtask 2: Isolate and culture mouse proximal tubule cells from CRT floxed mice	1-3	100%
Subtask 3: Validate CRT knockdown using human siRNA Cell lines used: HK-2 [ATCC CRL-2190]	3-6	100%
Subtask 4: Validate Cre Recombinase plasmid activity and CRT downregulation in transfected mouse proximal tubule cells	3-6	90%
Milestone(s) Achieved: Obtain IRB/HRPO approval. Validation of CRT knockdown protocols in human and mouse cells		
Major Task 2: (Aim 1.2) TGF-β and glucose response assays	**7-20**	**Murphy-Ullrich**
Subtask 1: measure ECM production in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	7-9	80% in HK-2 10% in mouse PTEC
Subtask 2: measure apoptosis in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	10-12	0%
Subtask 3: measure cell migration/invasion in response to TGF-beta and glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	**12-18**	**0%**
Subtask 4: epithelial plasticity in response to TGF-beta and glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	**19-20**	**0%**
Milestone(s) Achieved: Determine role of CRT in tubular epithelial behavior in response to TGF-beta or glucose treatment		
Major Task 3: (Aim 1.3) Role of CRT in TGF-β and glucose mediated calcium release and NFAT activity	**21-25**	**Murphy-Ullrich**
Subtask 1: Measure intracellular calcium release in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	**21-23**	**0%**
Subtask 2: Measure NFAT activity (nuclear localization, reporter assay) in response to TGF-beta or glucose with CRT knockdown	**24-25**	**0%**

Cells used: HK-2, CRT floxed mouse tubule cells		
Milestone(s) Achieved: Determine role of CRT in calcium and NFAT regulation downstream of glucose and TGF-beta		
Major Task 4: (Aim 1.4) Role of NFAT in CRT-regulated HK-2 responses	26-36	Murphy-Ullrich
Subtask 1: Use NAFT inhibitor any cellular responses determined from Major task 2 to be CRT-dependent Cells used: HK-2, CRT floxed mouse tubule cells	26-36	Post-doc
Milestone(s) Achieved: determination of role of NFAT in cellular responses regulated by CRT		
Major Task 5: (Aim 1.5) Role of CRT in regulating hydrogen peroxide-dependent increases in calcium release and NFAT activity	26-36	Murphy-Ullrich
Subtask 1: determine if catalase blocks TGF-beta and glucose stimulation of epithelial plasticity, ECM expression, calcium release, and NFAT reporter activity in cells with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	26-28	Post-doc
Subtask 2: measure hydrogen peroxide levels in TGF-beta and glucose stimulated cells +/- Smad inhibitor in cells with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	29-36	Post-doc
Milestone(s) Achieved: Delineation of pathways by which CRT regulates cellular responses to glucose and TGF-beta; publication of 1-2 peer reviewed papers		

Specific Aim 2: To determine the role of CRT in mouse models of diabetic nephropathy	**Months**	**GSU**
Major Task 1: (Aim 2.1) Drive proximal tubule specific and general Cre-recombinase expression in diabetic CRT-floxed mice (116 mice + breeders)	**1-24**	**Murphy-Ullrich/Sanders**
Subtask 1: Submit animal use request to UAB IACUC and DoD ACURO	1-2	100%
Subtask 2: Confirm sequence of PEPCK promoter plasmid and clone into CRE-recombinase GFP construct, validate in floxed mouse PT cells in vitro	1-2	0%
Subtask 3: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice	3-5	0%
Subtask 4: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (66 mice)	7-12	0%
Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	12-14	Murphy-Ullrich, Ailing Lu
Subtask 6: validate CRE-recombinase GFP construct in floxed mouse PT cells in vitro	12	100%
Subtask 7: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown	**13-14**	**100%**

in tubules by PCR and protein in B6D2F1 CRT floxed mice		
Subtask 8: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (50 mice)	**15-21**	**100%**
Subtask 9: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	**21-24**	**90%**
Milestone(s) Achieved: obtain IACUC/ ACURO approval, validation of model and ultrasound/microbubble plasmid delivery; determination of role of CRT on diabetic CKD; submission of manuscript		
Major Task 2: (Aim 2.b) CRT shRNA knockdown in the Akita model of diabetic CKD (50 mice)	**10-24**	**Murphy-Ullrich/Sanders**
Subtask 1: identify active mouse CRT siRNA; develop shRNA plasmid under U6 promoter, and validate CRT knockdown with mouse cells (MEFS, mouse proximal tubule cells) in vitro.	10-12	80%
Subtask 2: Validate CRT shRNA knockdown delivered via renal artery in pilot study (5 mice)	**12-14**	**0**
Subtask 3: Perform uninephrectomy; confirm diabetes in Akita mouse (45 mice)	**15**	**0**
Subtask 4: Deliver CRT shRNA via renal artery; repeat as necessary	**16-20**	**0**
Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	**21-24**	**0**
Milestone(s) Achieved: validation of CRT shRNA approach and determination of role of CRT in the Akita uninephrectomy model; submission of manuscript combined with Major Task 1 of Aim 2.		
Major Task 3: (Aim 2c) Determine the role of NFAT in diabetic nephropathy through use of a specific NFAT inhibitor (35 mice)	25-36	Murphy-Ullrich
Subtask 1: Perform uninephrectomy in Akita mouse; confirm diabetes	25	Sanders/Core B
Subtask 2: Deliver 11R-VIVIT peptide 3 x/week i.p. over 15 weeks	26-29	Ailing Lu
Subtask 3: : animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	30-36	Murphy-Ullrich, Ailing Lu, Core B
Milestone(s) Achieved: Determination of role of NFAT in tubulointerstitial fibrosis and renal function in the Akita uninephrectomy model; publish manuscript		

b. WHAT AS ACCOMPLISHED UNDER THESE GOALS

Milestones for Year 2 (months 12-14) are highlighted in bold and yellow

Specific Aim 1: to determine the role of CRT in mediating the fibrogenic effects of TGF-β and glucose in renal cells

Major Task 1 (Aim 1.1a, 1.1b)

Subtask 1: Confirm Not human subjects research for use of HK-2 cells through UAB IACUC and DoD HRPO

Cell lines used: HK-2 [ATCC CRL-2190]

Completed

Subtask 2: Isolate and culture mouse proximal tubule cells from CRT floxed mice
Completed, we now can routinely culture these cells

Subtask 3: Validate CRT knockdown using human siRNA
Cell lines used: HK-2 [ATCC CRL-2190]
Using electroporation, we showed that we can knockdown CRT mRNA (A) and protein (B) over a 96 hour period by delivering siRNA **(Figure 1)**. In these studies and in others not shown, maximum down regulation of CRT protein occurred between 48-96 hrs post siRNA transfection with typically 40-60% knockdown, which has been sufficient to see effects in previous studies with fibroblasts and vascular smooth muscle cells.

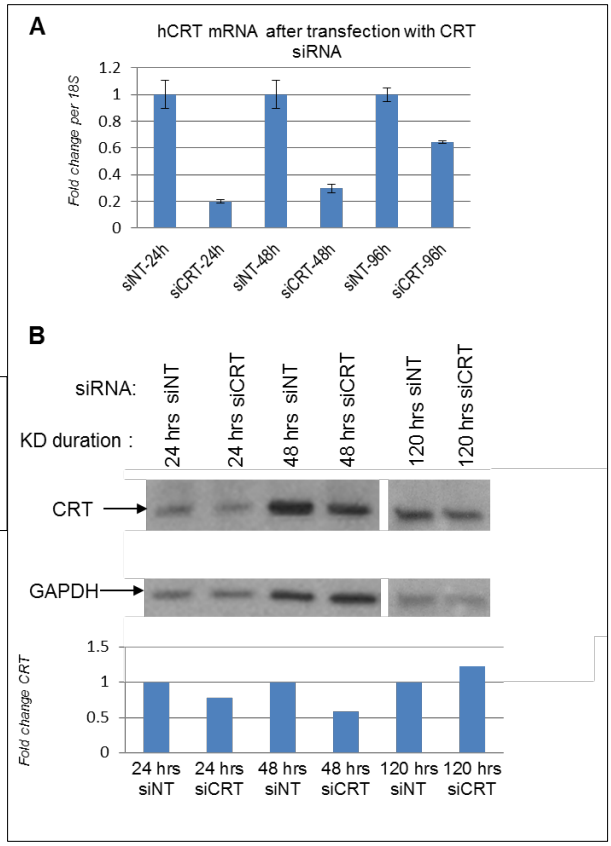
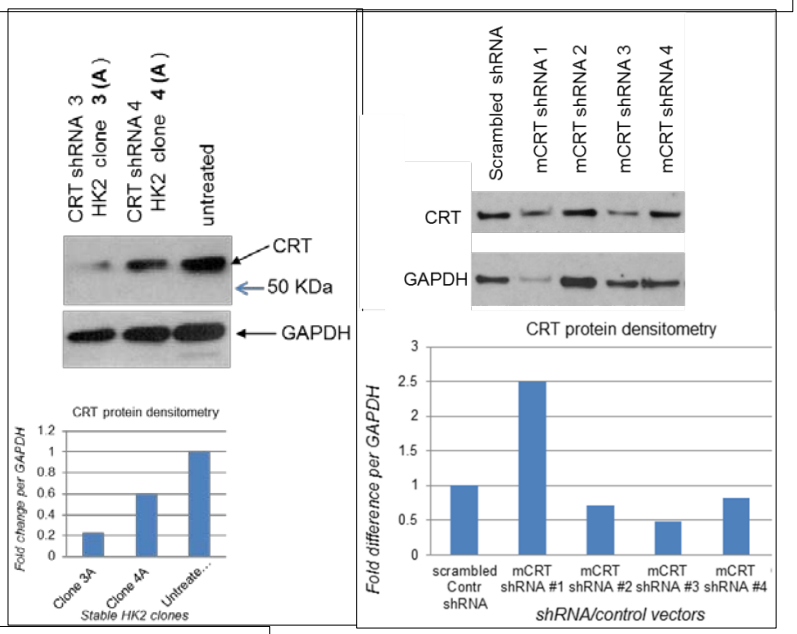
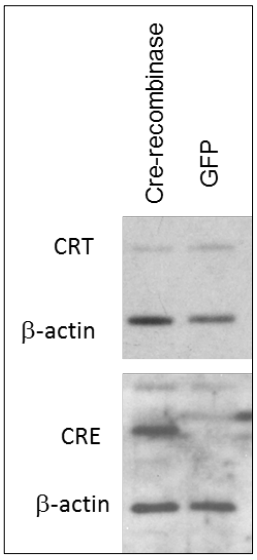


Figure 1: Knockdown of CRT message and protein in HK-2 cells by siRNA over time HK-2 cells were transfected with siRNA to CRT or with non-targeting siRNA (NT). (A) Message levels for CRT were measured by RTQ-PCR and (B) protein by western blotting with densitometric analysis following normalization to GAPDH.

In year 1, we identified 4 human CRT shRNA plasmids that reduce CRT expression in 293 HEK cells, transduced HK-2 cells and selected clones; we observed reduced CRT in 2 separate HK-2 clones **(Figure 2 left)**. In year 2, we selected the most robust shCRT and have isolated stably transduced HK-2 cells with reduced CRT expression **(Figure 9, page 10)**. Transduced cells were selected by FACS sorting for GFP expression. We have had some issues with cell viability in cells transduced with the non-targeting plasmid expressing lentivirus and we are currently regenerating this control HK-2 cell line. We will use these in year 3 to complete in vitro studies in Aim 1.

Figure 2. CRT shRNA reduces CRT expression in (A) HK-2 cells and (B) Mouse PTC cells.





Subtask 4: Validate Cre Recombinase plasmid activity and CRT downregulation in transfected mouse proximal tubule cells
We have been able to culture multiple isolates of primary mouse proximal tubular epithelial (PT) cells. As these are primary cells, transfection efficiency has been low (30-40%). We used both lipofectamine and electroporation approaches with similar results. Despite the low transfection efficiency, we were able to detect nearly 60% downregulation of CRT protein in mouse cells

Figure 3: Knockdown of CRT protein in primary mouse proximal tubule cells isolated from CRT floxed mice. Mouse proximal tubule cells were transfected with plasmids expressing Cre-recombinase-IRES-GFP (CRE) or with plasmid expressing GFP. After 72 hrs, cells lysates were analyzed for CRT protein and also for CRE to validate expression of the plasmid. Densitometric analysis showed a 60% reduction in CRT protein.

transfected with Cre-recombinase plasmid, but not with GFP control plasmid **(Figure 3)**. We also observed downregulation of CRT expression in vivo in mouse proximal tubules (see Major Task 2, Subtask 7).

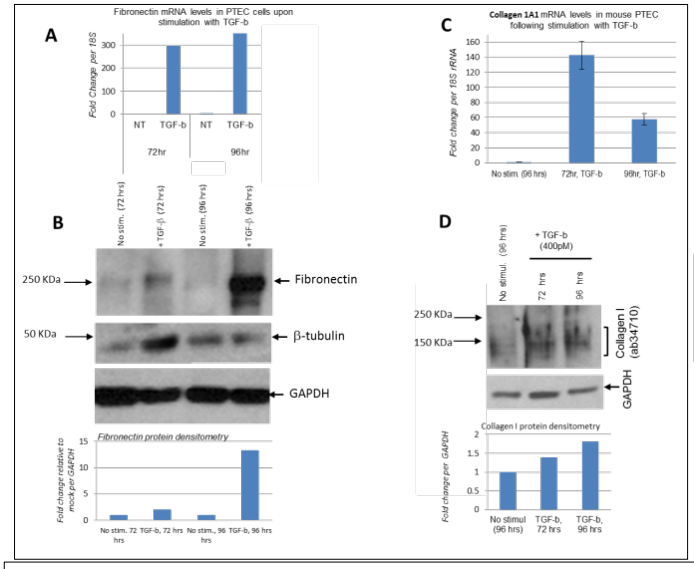
Major Task 2 (aim 1.2)

Subtask 1: measure ECM production in response to TGF-beta or glucose with CRT knockdown

Cells used: HK-2, CRT floxed mouse tubule cells

Studies showed that TGF-β stimulated fibronectin (FN) and type I collagen production in HK-2 cells at both the mRNA and protein levels **(Figure 4)**. Similarly, TGF-β stimulated FN and col I mRNA and protein expression by mouse PT cells **(Figure 5)**.

Figure 4: TGF-β stimulates fibronectin (FN) and collagen type I (col I) mRNA and protein expression by HK-2 cells.



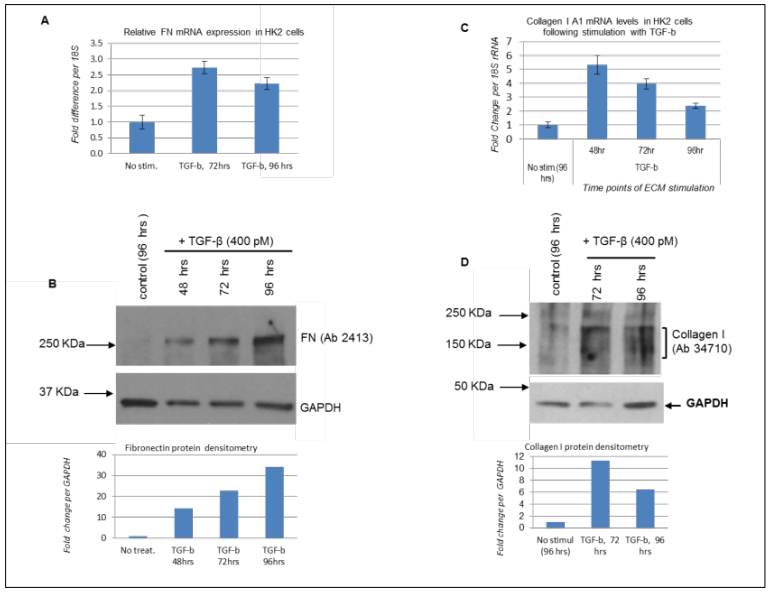
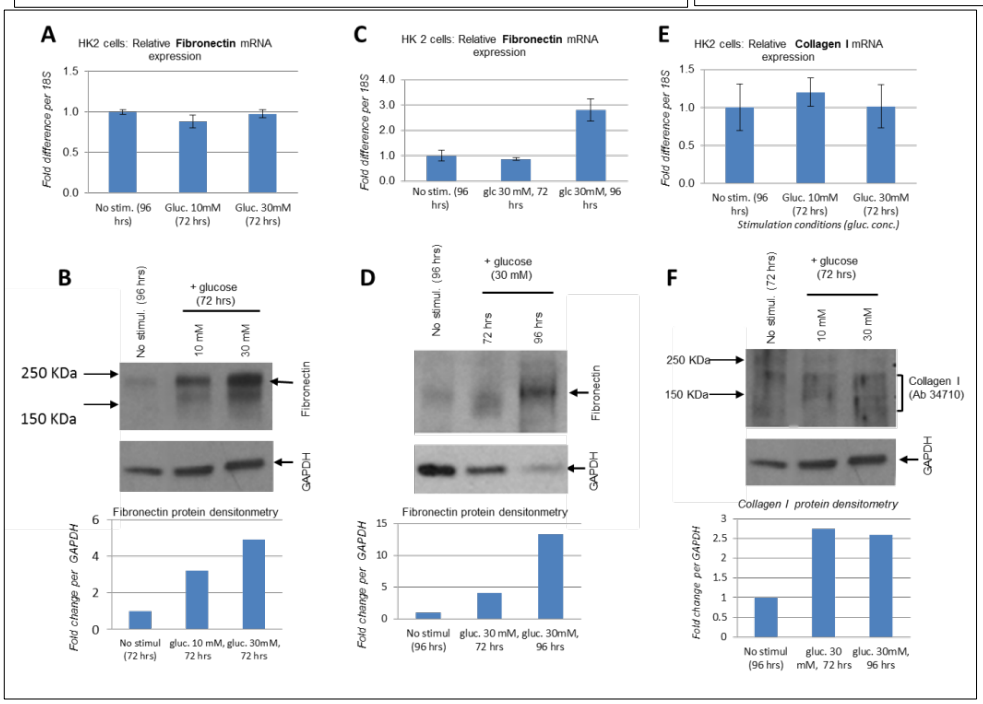


Figure 5: TGF-β stimulates fibronectin (FN) and collagen type I (col I) m RNA and protein expression by mouse proximal tubules cells isolated from CRT floxed mice.

Figure 6: Glucose (30 mM) stimulates FN and col I expression by HK-2 cells. HK-2 cells were grown in media with 6 mM glucose (control), 10 mM, or 30 mM glucose. 30 mM glucose stimulated increased FN mRNA and protein levels after72- 96 hrs of treatment. Col I protein was increased by 72 hrs with 30 mM glucose, although mRNA levels were not increased at this time point.



In further studies, we treated HK-2 cells and mouse PT cells with either 5.5-6 or 30 mM glucose and examined FN and type I collagen production. FN was increased by 30 mM glucose relative to 5.5 mM glucose (Mouse PTC) or 6 mM glucose (HK-2) [designated as no stimulation condition]. Glucose stimulated FN expression in HK-2 cells at 72-96 hrs, although glucose induction of FN was more transient in the mouse PTC cells, peaking at 72 hrs. Collagen I protein, but not message, was also stimulated by 30 mM glucose treatment of HK-2 cells at 72-96 hrs **(Figures 6,7)**.

To determine CRT's role in mediating TGF-beta and glucose-dependent ECM production, HK-2 cells were stimulated with TGF-beta following knockdown of CRT with siRNA **(Figure 8)** and collagen protein levels examined. CRT knockdown reduced TGF-beta stimulated levels of collagen I protein. In year 2, we also have data showing that CRT knockdown by siRNA reduces TGF-β and glucose stimulation of FN and TSP1 and we are optimizing conditions for looking at col IV (data not shown).

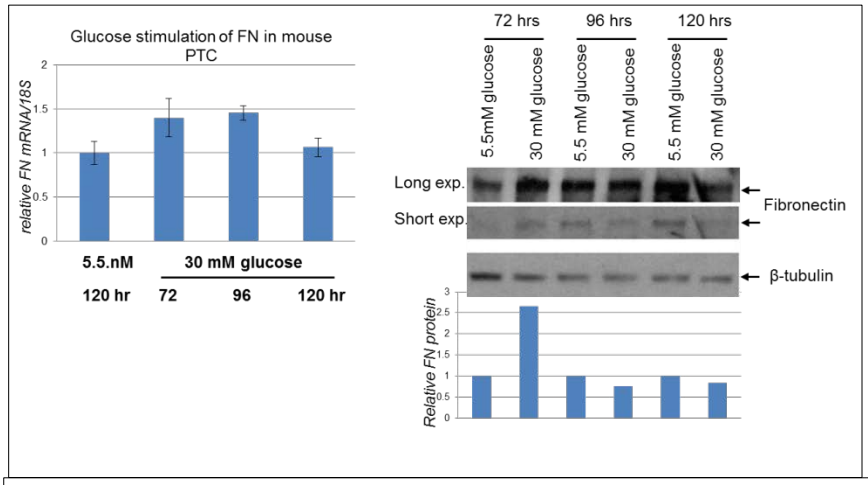


Figure 7: Glucose (30 mM) stimulates FN m RNA and protein expression by primary mouse PT cells. Mouse PT cells were grown in media with 5.5 mM glucose (non-stimulated control) or 30 mM glucose. 30 mM glucose stimulated increased FN mRNA and protein levels after 72 hrs of treatment.

Due to the variable transfection efficiencies, we have not been able to obtain reliable data with the mouse PT cells. We have identified several shRNA which are effective in mouse PT cells (see Major Task 2 (Aim 2.b, Subtask 1). Although, we expect that our lentiviral transduction approach to express CRT shRNA and selection of cells with stable CRT knockdown will increase the feasibility of these studies, the PTEC cells remain difficult to passage and we chose instead to focus on the HK-2 cells.

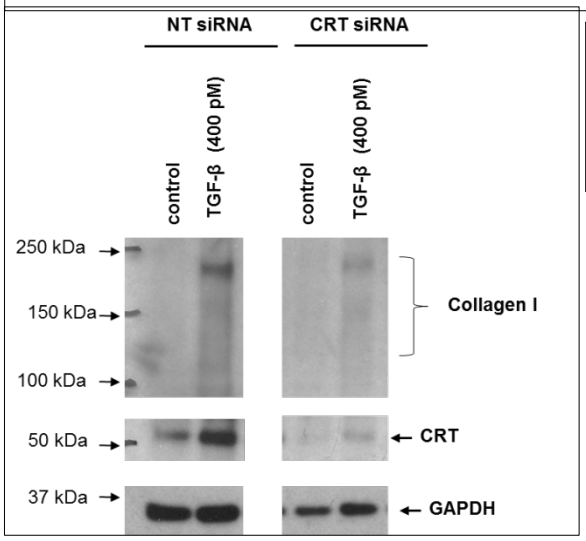


Figure 8: siRNA knockdown of CRT in HK-2 cells attenuates TGF-β stimulated col I protein expression. HK-2 cells were transfected with CRT or NT siRNA and 24 hrs later cells were stimulated with 400 pM TGF-β for 72 hr. Type I collagen levels were detected by western blotting of cell lysates. Blots also confirmed CRT knockdown.

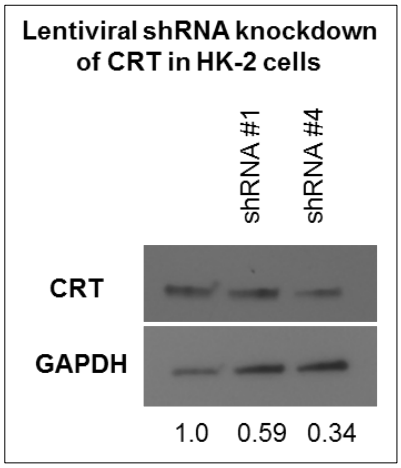


Figure 9: shRNA CRT knockdown through lentiviral transduction of HK-2 cells

Subtask 2: measure apoptosis in response to TGF-beta or glucose with CRT knockdown; Cells used: HK-2, CRT floxed mouse tubule cells

We did not make any progress on this task. Having the stably transduced shRNA CRT knockdown clones will increase the success of this aim in year 3.

Specific Aim 2: to determine the role of CRT in mouse models of diabetic nephropathy

Major Task 1 Aim 2.1

Subtask 1: Submit animal use request to UAB IACUC and DoD ACURO

Completed. We have obtained UAB IACUC and DoD ACURO approval.

Subtask 2: Confirm sequence of PEPCK promoter plasmid and clone into CRE-recombinase GFP construct, validate in floxed mouse PT cells in vitro

Subtask 3: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice

Subtask 4: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (66 mice)

Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)

Update from year 2

Subtasks 2-5: These tasks are related to the PEPCK promoter plasmid and confirmation of renal delivery have been delayed. e chose to proceed with the general knockdown approach first, because there is little glomerular expression of CRT. Most CRT expression is tubular, although the IHC suggests collecting duct and distal tubules express CRT as well as proximal tubules. In light of the success with the general knockdown approach, we do not think it is necessary to generate the PEPCK promoter driven Cre-recombinase.

Subtask 6: validate CRE-recombinase GFP construct in floxed mouse PT cells in vitro
These data are discussed under Major Task 1, subtask 4.

Subtask 7: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice

Year 1 We performed two different pilot studies to assess the feasibility of using the ultrasound/microbubble (US/MB) approach to deliver cre-recombinase plasmid to the kidneys of floxed mice. Although we had previously established the efficacy of this approach in a mouse carotid artery ligation model (Zimmerman et al, manuscript in revision), we had not yet established efficacy and toxicity in the kidney.

ELISA for kim-1

Urine	Ear tag number	days	24h Urine µl	d2 pg/ml AVERAGE	d7 pg/ml AVERAGE
Control	914	d7	765		2955.73
No ultrasound	917	d7	1000		2757.37
Group1	903	d2	540	1185.72	
0.2 MPa	913	d7	530		4344.06
	924	d7	650		2893.09
Group2	898	d2	315	6598.23	
0.7MP2	912	d7	119		3884.80
Group3	896	d2	410	7636.46	
1.3MPa	904	d7	355		3081.01
	905	d7	1210		1331.96

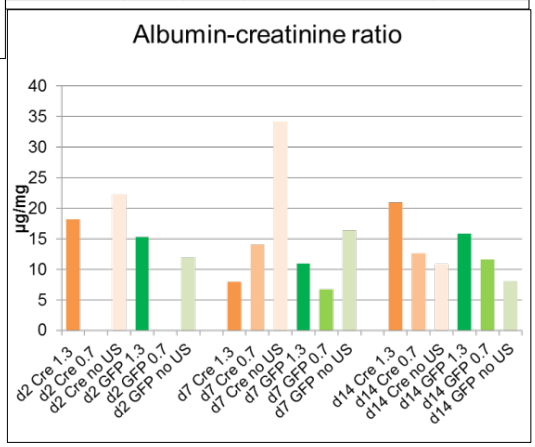


Figure 10: Analysis of renal damage from urinary markers in MB/US treated mice. 24 hr urines were collected from mice after treatment with various US strengths and analyzed by ELISA for KIM-1, a marker of acute kidney injury, and for urinary albumin and creatinine, expressed as the ACR or albumin/creatinine ratio. US treatment did not increase detectable markers of renal damage.

In the first pilot study, we treated mice (n=2-3/group) with 3 different intensities of US and delivered microbubbles without plasmid. Mice were injected with 200 µl MB, rapidly anesthetized via isofluorane inhalation, and then the dorsal kidney areas were subjected to 0.2, 0.7, or 1.3 MPa of ultrasound pressure for a total of 1 min exposure, consisting of two 30 sec pulses with a 30 sec interval. Mice were harvested on days 2, 7, and 14 after MB/US treatment and 24 hr urines collected prior to sacrifice. Urines were analyzed for early (kim-1) and later markers of renal injury (albumin, creatinine). Hematoxylin & Eosin-stained kidney sections were also examined for signs of morphologic damage. Histologic analyses showed some tubular damage at the outer cortical regions of the kidney, especially in 1.3 MPa treated mice, and to a lower extent in the reduced US dosed mice (0.2, 0.7) (data not shown). However, the urinary analyses did not show elevations in kim-1, a marker of acute kidney injury, or in urinary albumin or creatinine **(Figure 10)**.

In the second pilot study, we evaluated the efficacy of MB/US-mediated targeted cre-recombinase plasmid delivery to the kidney. The cre-recombinase plasmid was shown to have activity in reducing CRT expression of mouse PT cells isolated from the CRT floxed mice (Major task 1, Subtask 4, Figure 3). We used 3 animals/group. Mice were 8-17 weeks of age and of both sexes. Mice were treated via tail vein injection with 300 µg of plasmid in 50 µl of saline, which was combined with 200 µl of Optison MBs as described for pilot study 1. Plasmids were either GFP or Cre-recombinase-IRES-GFP and were validated in our previous mouse carotid studies and in the mouse PT cells. Anesthetized animals were subjected to either 0.7 or 1.3 MPa of ultrasound as described in pilot study 1. Kidneys were harvested from animals at days 2, 7, and 14 following MB/US treatment/plasmid delivery. Kidneys were analyzed for CRT mRNA by RT-PCR (1.3 MPa dose only), CRT protein in renal lysates by western blotting, and staining for CRT in kidney sections by immunohistochemistry and morphometric analyses. Interestingly, the higher dose US treatment (1.3 MPa) did not significantly knockdown CRT expression at either the mRNA or protein level in the kidneys (data not shown). However, we did observe downregulation of CRT protein in renal lysates and by IHC morphometric analyses in the kidneys from mice treated with 0.7 MPa of US **(Figures 11A-C)**. Surprisingly, CRT protein knockdown occurred fairly rapidly (by 2 days), but persisted over 14 days. Note that CRT expression is localized primarily to the tubules and that MB/US delivery of cre-recombinase downregulates CRT expression in the tubules. We interpreted the response with 0.7 MPa US vs 1.3 MPa US to reflect a balance between US-mediated induction of CRT (due to cellular

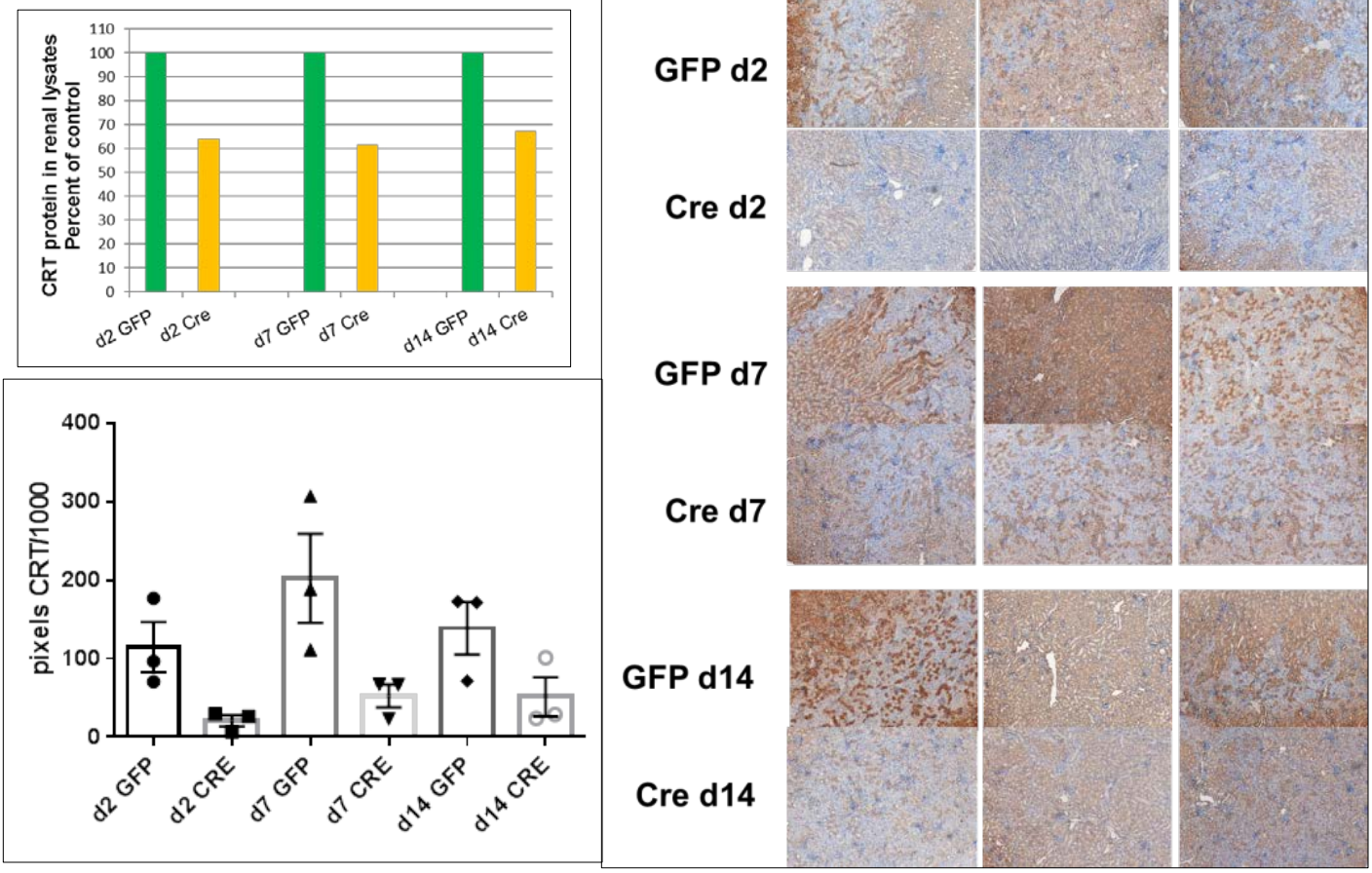


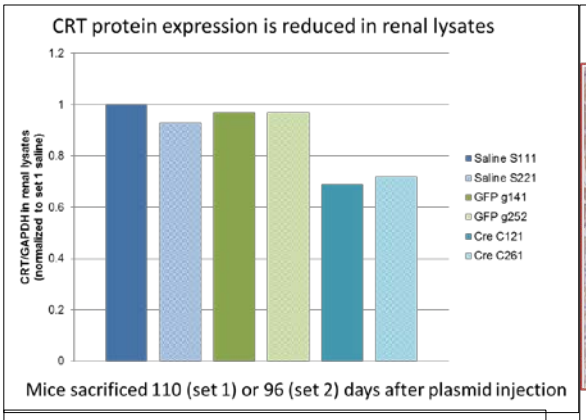
Figure 11. Delivery of Cre-recombinase plasmid (CRE) with microbubbles (MB) to CRT floxed mice via kidney-targeted Ultrasound (US) reduces CRT expression in kidneys. (A) CRT protein was reduced in renal lysates as detected by western blotting of renal tissue in mice receiving Cre-recombinase plasmid (CRE), but not in mice given control GFP plasmid. **(B)** Immunostaining for CRT using a rabbit monoclonal antibody to CRT on kidney sections from treated mice shows expression of CRT in renal tubules and reduced tubular CRT expression in mice receiving Cre-recombinase plasmid (original mag 10x). **(C)** Morphometric analysis of CRT immunostaining in renal sections from mice harvested at days 2, 7, and 14 following ultrasound treatment (n=3)/group, error bars are SEM.

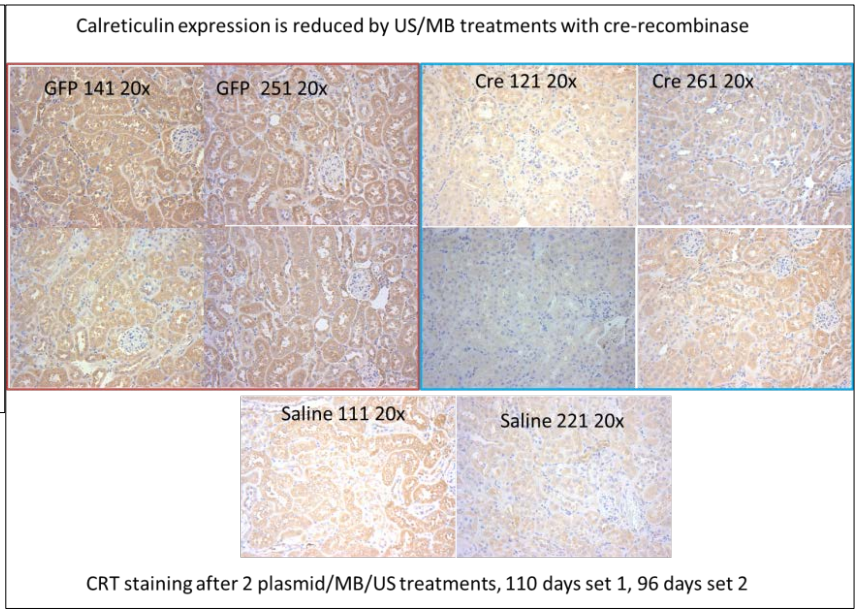
stress and/or mechanotransduction) and the ability of cre-recombinase to excise the CRT gene to reduce protein expression. Because of the predominance of CRT expression in the tubules, we have decided to proceed directly with animal studies without first generating the tubule specific PEPCK promoter constructs. Note that we are able to use tail vein delivery instead of direct delivery through the renal artery, which eliminates a surgical procedure.

Year 2 Accomplishments

Subtask 8: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks

In year 2, CRT floxed mice were subjected to uninephrectomy and then 2 weeks later, diabetes was induced with repeated injections of low dose streptozotocin (STZ). Only mice with blood glucose levels of >250 were used for further studies. Sonoporation with ultrasound (0.7 MPa) was performed twice, 2 and 4 weeks after STZ treatment. 2 animals from each group (saline injection, but no US or microbubbles (US/MB), GFP plasmid with US/MB treatment as a control for US-mediated renal injury, and Cre-recombinase plasmid with US/MB) were sacrificed 3-3.5 months after the first treatment to assess long-term down-regulation of CRT in the kidney. Pilot studies done in year 1 showed CRT downregulation at 7-14 days post-treatment, but the long-term stability of CRT downregulation was unknown. These studies showed that CRT expression remained reduced to about 70% of controls at 14-16 weeks post-treatment as measured by immunoblotting of renal lysates and by IHC staining for CRT in kidney sections **(Figure 12a,b)**. Similar analyses of renal tissues at the termination of the studies at 22 weeks showed sustained reduction in renal CRT to~70% of control levels **(Figure 12c)**. Note that the tubules stain strongly for CRT and most of the reduction in CRT occurs in the tubules.





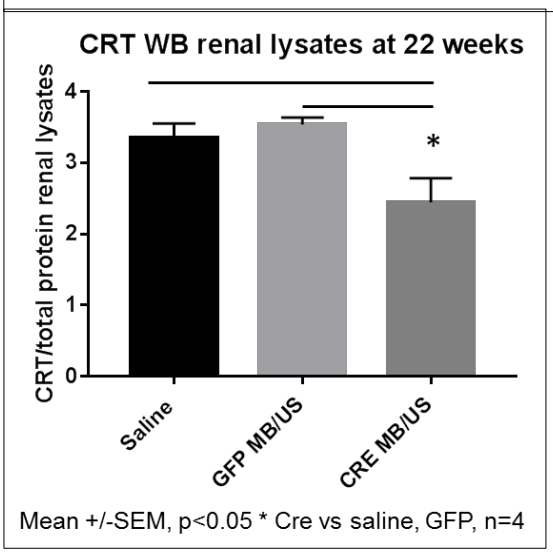
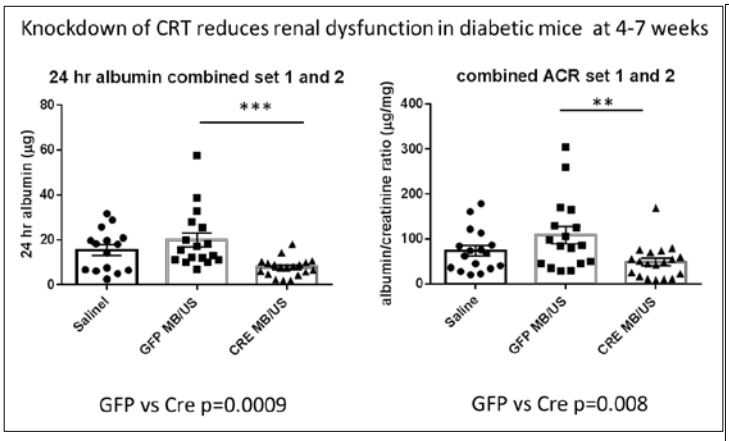


Figure 12: Analyses of CRT knockdown by Cre-recombinase plasmid and MB/US in kidneys at 14-16 weeks and at 22 weeks.

Subtask 9: animal model analysis (histology, EM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers

Year 2 To assess the effect of CRT knockdown on renal function in the diabetic kidney, we measured both urinary albumin and creatinine and serum creatinine. Urine measurements were performed both at early disease (2-7 weeks with some collections at 11-12 weeks) and at late disease (17-19 weeks). At the early time point, the urinary albumin and albumin/creatinine ratio were significantly reduced in mice treated with cre-recombinase plasmid to knockdown CRT as compared to GFP-plasmid controls **(Figure 13a)**. In late disease, animals with Cre-recombinase treatment also had reduced urinary albumin and albumin/creatinine ratio as compared to both saline control and GFP-treated mice **(Figure 13b**). Serum creatinine levels were unaffected by treatment (data not shown). Importantly, knockdown of CRT also improved survival of the diabetic mice ($p<0.07$)**(Figure 14).** Renal sections from these mice were also examined by electron microscopy and PAS, fibronectin, and type IV collagen staining for matrix. PAS staining shows a reduction in both glomerular and peri-tubular basement membrane staining in Cre-recombinase treated mice **(Figure 15)**. Consistent with the PAS staining, electron microscopic observation of renal tissues showed extensive tubulointerstitial collagen and glomerular basement membrane and mesangial thickening in diabetic mice treated with GFP control plasmid. This damage was reduced in Cre-recombinase treated kidneys **(Figure 16)**. Renal lysates were also immunoblotted for fibronectin and type IV collagen. Fibronectin and collagen IV levels were all decreased in samples from mice treated with Cre-recombinase (data not shown)**.** Although quantitative analyses of the immunohistochemistry are not yet completed, these factors are also decreased. Sections will also be stained and analyzed for nuclear NFAT to determine if reduction in CRT expression reduces NFAT activation.



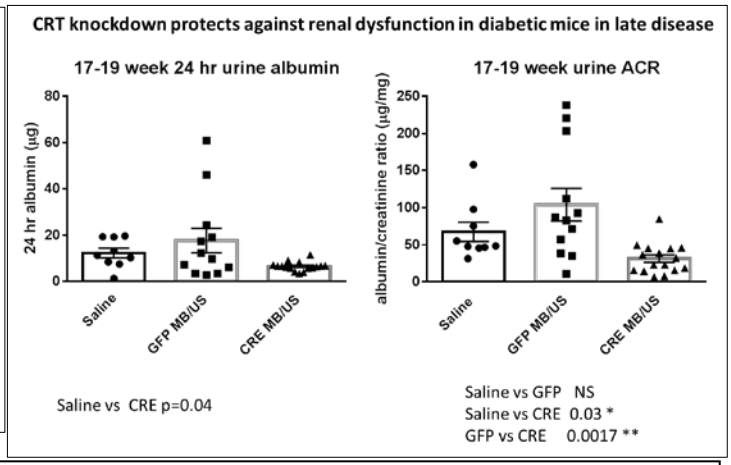


Figure 13: urinary albumin and creatinine measurements

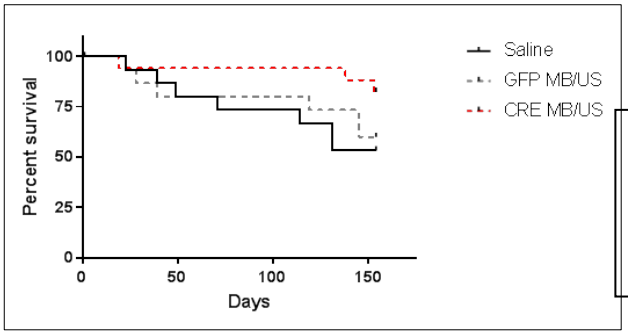


Figure 14: CRT knockdown improves survival of diabetic mice Mantel-Cox (logRank) analyses of animal survival shows a benefit (p0.07) in mice with cre-recombinase MB/US knockdown of CRT (82% at 154 days) as compared to saline (53%) or GFP MB/US (60%) groups. Mice which died on the day of MB/US treatment (GFP 2, Cre 1) were censored.

The results of these studies are important, because they show that 1) we can effectively and stably knockdown CRT expression in the kidney using targeted ultrasound delivery of cre-recombinase plasmid to CRT floxed mice and 2) that CRT knockdown attenuates renal dysfunction and fibrosis and improves survival in diabetic mice. These studies validate our overall hypothesis.

Major Task 2 (Aim 2.b)

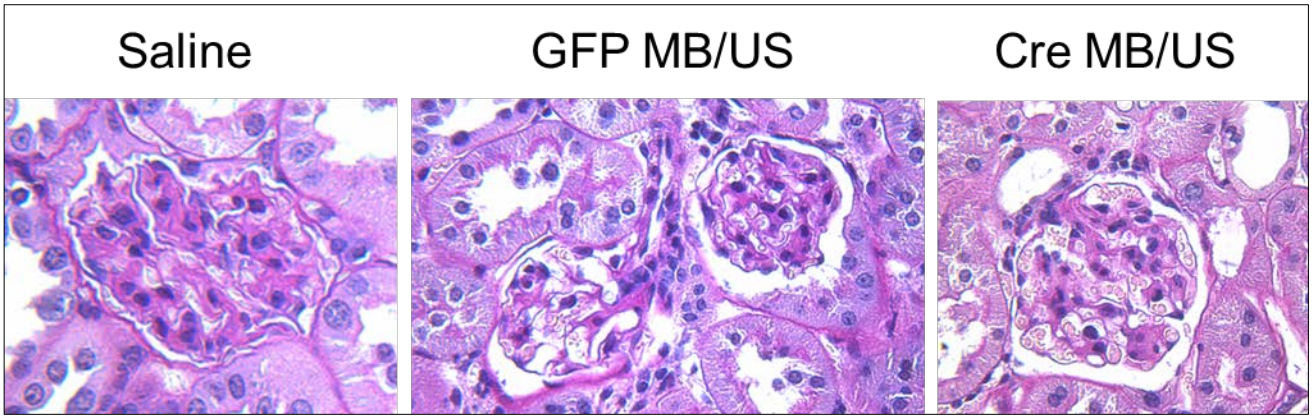


Figure 15: PAS staining of kidneys Saline and GFP MB/US treated diabetic mice showed increased mesangial matrix and thickened glomerular basement membrane and tubular basement membrane staining as compared to Cre MB/US diabetic mice. Note the cellular lysis in one of the glomeruli from the GFP MB/US mouse. 40x original magnification.

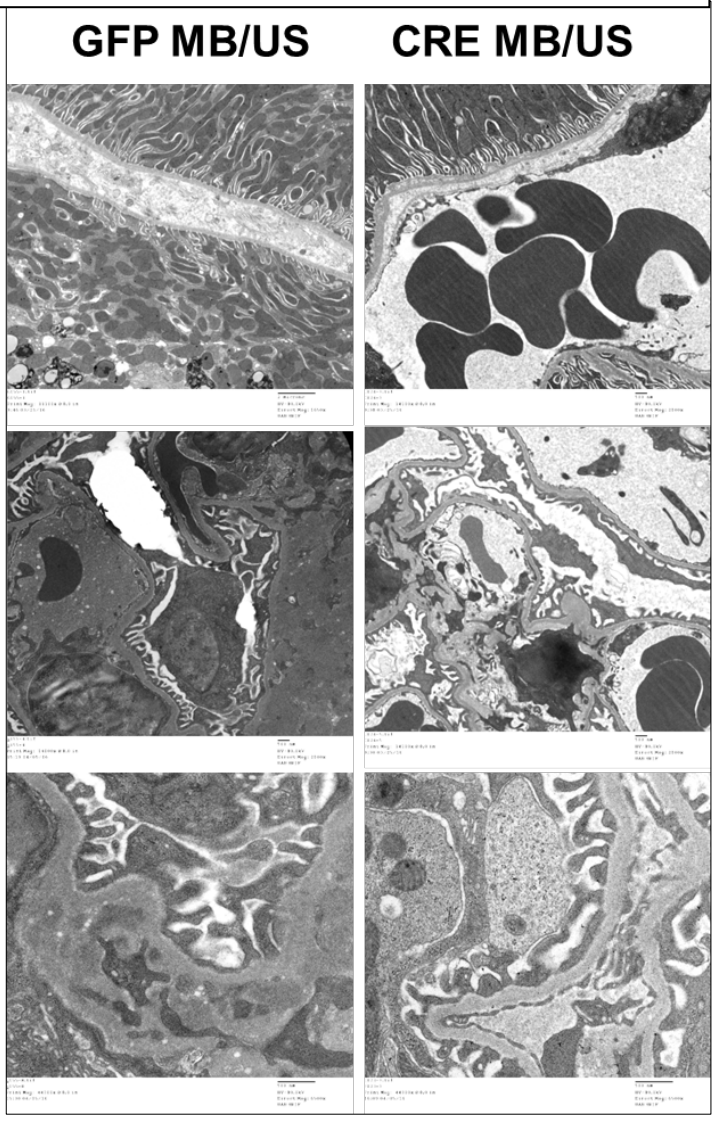


Figure 16: Electron microscopy of renal sections from diabetic mice. Two 2 panels show proximal tubules and middle and bottom panels show glomerular regions.

Subtask 1: identify active mouse CRT siRNA; develop shRNA plasmid under U6 promoter, and validate CRT knockdown with mouse cells (MEFS, mouse proximal tubule cells) in vitro.

In late year 1, we identified multiple shRNAs to knockdown mouse CRT **(Figure 2 right)** and packaged this shRNA into a lentivirus to be used in the Akita model. However, given the success we achieved in Year 2 using the STZ-treated CRT-floxed mice, we feel that repeating these studies in the Akita mice with lentiviral CRT shRNA delivery is redundant and that these studies will not provide substantial new knowledge, given the cost and effort it requires. Rather, we intend to proceed with the NFAT inhibitor studies using the CRT floxed mice. This will permit a more direct comparison between the effects of CRT knockdown and NFAT inhibition using the same model system.

Summary of Key Research Accomplishments

During **year 1**, we established that we can culture mouse proximal tubule cells; knockdown CRT, either via siRNA or shRNA; showed the kinetics of TGF-beta and glucose stimulation of FN and collagen I; and have preliminary data that CRT knockdown attenuates ECM production in response to TGF-beta stimulation. We also established the feasibility of renal knockdown of CRT in the pilot studies and established that markers of renal injury are not elevated by US/MB treatment. Although many of these in vitro studies will need to be repeated prior to publication, these initial data support our hypothesis and justify further in vitro studies as outlined in the proposal.

In year 2, we accomplished the following:

Aim 1: We generated stably transduced lines of HK-2 cells with CRT knockdown to be used to complete Aim 1 studies. Although we identified mouse CRT shRNA constructs for use in mouse PTEC, we continued to have difficulties in passaging these cells, which is necessary for clonal selection and expansion of cultures for subsequent studies. We also pursued further studies with siRNA knockdown for examination of effects on TGF-beta and 30 mM glucose stimulation of ECM components (FN, TSP-1, col I, col IV). For year 3, we will focus on completing studies with HK-2 cells with stable CRT knockdown.

Aim 2: The results of these studies are particularly encouraging to us. We showed that we can induce sustained knockdown of CRT in the kidney, primarily in the tubules, using targeted US/MB delivery of cre-recominbase over the duration of these studies (22 weeks). Importantly, we showed that knockdown of CRT attenuated renal dysfunction and fibrosis in the diabetic kidney and improved survival, providing essential data to support our hypothesis.

c. WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

There was no specific intent to provide training or professional opportunities in this grant.

d. HOW WERE THE RESULTS DISSEMINATED?

Although nothing was disseminated in year 2, we have plans to start reporting the findings at meetings in year 3 and begin preparation of a manuscript to be submitted in year 3. For year 3, we have submitted an abstract for a poster presentation at the 2016 American Society for Matrix Biology meeting in St. Petersburg, FL Nov 13-16, 2016. The PI will be presenting this work at the Calreticulin Workshop in Delphi, Greece May 18-21, 2017.

e. WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

During year 3, we will optimize the lentiviral-mediated knockdown of CRT and the non-targeting shRNA control in the HK-2 cells. We will complete the cell based studies in Aim 1.

In specific Aim 2, we will complete the analyses of the tissues from the year 2 animal studies. We will perform the studies using the 11R-VIVIT inhibitor in the CRT floxed mice treated with STZ and uninephrectomy, and complete the analyses.

We will complete all studies and prepare a manuscript for submission.

4. IMPACT

a. WHAT WAS THE IMPACT ON THE DEVELOPMENT OF THE PRINCIPAL DISCIPLINES OF THE PROJECT? These studies confirm the importance of calreticulin signaling on the development of fibrosis in diabetic nephropathy. This is a novel finding and in year 3 we intend to address the mechanisms utilized by calreticulin to drive fibrosis in the diabetic kidney.

b. WHAT WAS THE IMPACT ON OTHER DISCIPLINES? These studies provide additional support for the use of targeted ultrasound with microbubbles to selectively knockdown gene expression in targeted organs. This approach takes advantage of available "floxed" mice and eliminates the need for developing new transgenic strains with inducible and/or cell type-specific cre strategies.

c. WHAT WAS THE IMPACT ON TECHNOLOGY TRANSFER? Nothing to report

d. **WHAT WAS THE IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY?** These studies identify a new target for the treatment of diabetic nephropathy. Studies with the NFAT inhibitor 11R-VIVIT will provide further insights into the potential for use of calreticulin blocking strategies and/or NFAT inhbitors to treat chronic kidney disease in diabetes.

5. CHANGES/PROBLEMS

a. CHANGES IN APPROACH AND REASONS FOR CHANGE

In our pilot studies, we learned that most of the detectable CRT in the mouse kidney is in the tubules. Delivery of cre-recombinase via US/MB mediated delivery through the tail vein predominantly down-regulated CRT expression in the tubules (proximal, distal, and collecting ducts). Glomerular CRT under control and treated conditions was nearly undetectable. In the animal studies performed in **year 2**, we showed that this approach was sufficient to reduce renal dysfunction and fibrosis, making the studies using the PEPCK-tubule specific promoter to deliver the cre-recombinase plasmid to the proximal tubules unnecessary. Instead in year 3, we will focus on linking NFAT activity to diabetic nephropathy through the use of the cell permeable NFAT inhibitor 11R-VIVT in animal studies. The drug has been ordered and we expect to begin these studies in early fall (Major task 3, Aim 2c). In the next few months, we will complete our analyses of NFAT and TGF-beta signaling in the kidneys from the year 2 studies. These data will link CRT downregulation to the role of NFAT in diabetic nephropathy. Importantly, we will perform these studies in the STZ-treated CRT floxed mice rather than in the Akita mice. The data we obtained in year 2 confirms the importance of CRT in diabetic renal fibrosis and renal function and by performing the 11R-VIVIT studies in this same model, we will be able to more directly compare the effects of CRT knockdown to those of NFAT inhibition. Choosing this model will eliminate any strain specific differences in diabetic nephropathy progression between the Akita model and the CRT floxed B6 x DBA/2J strain. We believe that this is the most efficient means to accomplish our goals.

For in vitro studies, we had initially proposed using siRNA transient transfection via electroporation. Although this works for the HK-2 cells, transfection of the primary mouse proximal tubule cells was less efficient. We also saw that CRT knockdown via siRNA began to lose its effectiveness at 96 hrs. This shortened knockdown period could present a problem with some of the more delayed effects of glucose stimulation. To obtain more homogeneous populations with more stable knockdown, we switched to using a lentivirus expressing shRNA for CRT. This approach has been successful in generating HK-2 cells with stable CRT knockdown. The shRNA plasmid has both GFP expression and puromycin resistance genes. Flow cytometry was used to select and enrich HK-2 populations with stable knockdown of CRT for use in the longer assays in Aim 1. Although we need to select another non-targeting shRNA control, we fully expect the cell-based studies in Aim 1 to be completed by the end of year 3.

b. ACTUAL OR ANTICIPATED PROBLEMS OR DELAYS AND ACTIONS OR PLANS TO RESOLVE THEM

In year 2, we focused on the animal studies in aim 2. We did not have any technical issues with these studies. Fortunately, the targeted ultrasound knockdown of CRT was stable over the 22 week course of the studies and therefore, we only had to perform 2 treatments at the initiation of the model, rather than the anticipated treatments every 3 weeks during the entire course of the studies. This served to minimize the renal damage to ultrasound treatments, making this model more reliable.

In studies in Aim 1, we were able to generate stably transduced CRT knockdown HK-2 cells using lentivirus transduction of CRT shRNA. We used sorted transduced cells using GFP expression (on the shRNA plasmid) and maintain selection using puromycin. For reasons we do not understand, our attempts to make control HK-2 cells with non-targeting shRNA have been toxic to the cultures. We are starting with both fresh lentivirus and fresh HK-2 cultures to make these controls.

We also were able to knockdown mouse CRT using shRNA, however, the mouse PTEC remain difficult to passage. Even the stable transduction requires culture expansion and cultures start to senescence by the time we achieve stable clones. We believe that it will be more productive to focus on the HK-2 cell lines which have stable CRT knockdown for the Aim 1 in vitro studies and we are going to focus here. Discuss mouse shRNA and mouse PTEC

CHANGES THAT HAD A SIGNIFCIANT IMPACT ON EXPENDITURES

Anton Borovjagin moved to a different position at UAB that gave him more stable long-term funding and a clearer career path. He left this project on December 28, 20115. Mr. Antonio Pallero, who has handled cell culture and cell-based studies in my lab moved to this project with 75% effort on January 1, 2016. He generated the preliminary data for the grant and is familiar with the cell systems. His salary is substantially higher than Dr. Borovjagin's and therefore total expenditures for this position were not significantly affected. There was an initial lag in spending for Aim 1 in early 2016, although spending and execution of studies for this aim are fully underway. Primary expenditures were focused on the animal studies in Aim 2. In year 3, due to new FLSA regulations, Ailing Lu will be promoted and she will receive a pay equity salary adjustment. We anticipate heavy spending in year 3 to complete all the cell based studies in Aim 1 and the remaining animal studies and analyses in Aim 2.

c. SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS, VERTEBRATE ANIMALS, BIOHARZARDS, AND/OR SELECT AGENTS

i. SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS not applicable

ii. SIGNIFICANT CHANGES IN USE OR CARE OF VERTEBRATRE ANIMALS SIGNIFICANT

There are no changes from year 1. We received our annual UAB IACUC renewal on 11 February 2016.

iii. CHANGES IN USE OF BIOHARZARDS AND/OR SELECT AGENTS none

6. PRODUCTS

a. PUBLICATIONS, CONFERENCE PAPERS, AND PRSENTATIONS

i. JOURNAL PUBLICATIONS none

ii. BOOKS none

iii. OTHER PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS none

b. WEBSITE OR OTHER INTERNET SITES none

c. TECHNOLOGIES OR TECHNIQUES none

d. **INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES** none

e. OTHER PRODUCTS none

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. WHAT INDIVIDUALS WORKED ON THE PROJECT?

Name:	Joanne Murphy-Ullrich	Paul Sanders	Ailing Lu	Anton Borovjagin	Weiqi Lei	Manuel Antonio Pallero
Project Role:	PI	collaborator	Research associate	Research Associate	technician	Lab supervisor
Research Identifier	emurph (eCommons)	psanders (eCommons)	none	aborov (eCommons)	none	none
Person months worked	3.0	1.2	12	6 (100% for 6 months)	0	4.5 (75% for 6 months)
contribution	Oversight of the entire project, IACUC	Consultation on possible inducible	animal studies and analyses (IHC,	Mouse and human cell culture, siRNA,	Supervise breeding and phenotyping of CRT-	Mouse and human cell culture, siRNA,

	compliance, personnel supervision	Cre models specific for proximal tubule	western blot), renal injury markers	lentivirus sRNA generation/validation; TGF-beta and glucose analysis	floxed mice to C57bl/ background (part of general lab responsibilities)	lentivirus sRNA generation/validation; TGF-beta and glucose analysis
Funding support	NA	NA	NA	NA	NIH/NCI 1R01CA175012	25% effort NIH/NCI 1R01CA175012

b. HAS THERE BEEN A CHANGE IN THE ACTIVE OTHER SUPPORT OF THE PD/PI OR SENIOR/KEY PERSONNEL?

Murphy-Ullrich
(Grant support completed since 7/2015)
Eyesight Foundation of Alabama (38-2009-633) (Downs PI)
12/31/13-12/30/15 (annual direct) 0.60 cal months
(Murphy-Ullrich, PI subproject)
Role of TSP1-TGF-beta in biomechanical remodeling in glaucoma
Goals: To examine the role of TSP1 control of cell adhesion and TGF-beta activation in sclera remodeling under differing biomechanical forces in models of glaucoma.
Overlap: None
Contact: Torrey V. A. DeKeyser, Executive Director, Shirley Hamilton, Director of Grants and Programs, Eyesight Foundation of Alabama Board of Trustees, 700 South 18th Street, Suite 123, Birmingham, AL 35233 205/488-0771 Phone 205/325-8335 Fax

Paul Sanders
New Support
1 R01 HL126903-01A1 (PI: Agarwal, R) 12/15/2015 – 12/31/2020 Effort: 3.0 calendar
National Institutes of Health Annual direct costs:
ChLorthalidone In Chronic Kidney Disease (CLICK) Study
This double-blind, two-center, placebo-controlled, randomized trial will test the hypothesis that chlorthalidone will improve BP among subjects with advanced CKD (stages IV and V) and poorly controlled hypertension. The results of this trial will allow the safe and effective use of chlorthalidone among people with CKD and hypertension.

1U54TR001368 (PI: Kimberly) 09/01/2015 – 08/31/2020 Effort: 0.6 calendar
National Institutes of Health/NCATS Annual direct costs: (UL1, KL2, TL1)
UAB Center for Clinical and Translational Science (CCTS)
The UAB CCTS will enhance human health by driving scientific discovery and dialogue across the bench, bedside and community continuum. The CCTS support this overall mission in a highly integrative network of relationships. Success in creating such an environment is dependent upon success in achieving five strategic priorities: 1) enhancing research infrastructure; 2) promoting investigator education, training and development; 3) accelerating discovery across the T1 interface; 4) expanding value-added partnerships; and 5) building sustainability.

c. WHAT OTHER ORGANIZATIONS WERE INVOLVED AS PARTNERS? None

8. SPECIAL REPORTING REQUIREMENTS Not applicable, None

9. APPENDICES none